VIROLOGY 210, 273–282 (1995)

Characterization of the Regulatory Function of the ICP22 Protein of Equine Herpesvirus Type 1

V. ROGER HOLDEN, YUHE ZHAO, YEINI THOMPSON,* GRETCHEN B. CAUGHMAN,* RICHARD H. SMITH, and DENNIS J. O'CALLAGHAN[1]

*Department of Microbiology and Immunology, Louisiana State University Medical Center, 1501 Kings Highway, Shreveport, Louisiana 71130-3932; and *Department of Oral Microbiology, School of Dentistry, Medical College of Georgia, Augusta, Georgia 30912-1126*

Received February 6, 1995; accepted April 24, 1995

The IR4 gene (inverted repeat gene 4) of equine herpesvirus type 1 (EHV-), the homolog of the herpes simplex virus type 1 ICP22 gene, is differentially expressed as a 1.4-kb early transcript and a 1.7-kb late transcript that encode a series of proteins that migrate between 42 to 47 kDa, localize to the nucleus of EHV-1-infected cells, and become packaged within EHV-1 virions (V. R. Holden, G. B. Caughman, Y. Zhao, R. N. Harty, and D. J. O'Callaghan, *J. Virol.* 68, 4329–4340, 1994). To assess the role of the IR4 protein in EHV-1 gene regulation, an IR4 expression vector was cotransfected with EHV-1 chimeric promoter–CAT reporter constructs and EHV-1 effector plasmids to determine the effects of the IR4 protein on the expression of immediate-early (IE), early, and late promoters. These studies revealed that the IR4 protein: (i) minimally *trans*-activates EHV-1 promoters, (ii) acts synergistically with the UL3 (ICP27) gene product to *trans*-activate the IE promoter, (iii) does not interfere with the *trans*-repression of the IE promoter by the IE protein, (iv) enhances transactivation of early promoters by the IE protein, (v) enhances the transactivation of both early and late promoters by the IE and UL3 proteins, and (vi) interacts synergistically with the IE protein to *trans*-activate the heterologous HSV-1 ICP4 promoter. These data suggest that the IR4 gene product plays a significant role in EHV-1 gene regulation. © 1995 Academic Press, Inc.

INTRODUCTION

Equine herpesvirus type 1 (EHV-1), an α-herpesvirus, is a major pathogen of equines, causing respiratory disease, neurological disorders, and spontaneous abortions in pregnant mares (Allen and Bryans, 1986; Bryans and Allen, 1986; O'Callaghan *et al.*, 1968). Previous work has determined that EHV-1 encodes at least 77 ORFs (Gray *et al.*, 1987a,b; Holden *et al.*, 1992a; Telford *et al.*, 1992) which are expressed in a coordinately regulated and sequentially ordered fashion as immediate-early, early, and late genes (Caughman *et al.*, 1985; Gray *et al.*, 1987b). Several of the 77 ORFs are known to encode proteins that are involved in EHV-1 gene regulation. ORF12 of EHV-1 exhibits homology to the herpes simplex virus type 1 (HSV-1) α-TIF protein (Telford *et al.*, 1992), and its expression elevates the expression of the EHV-1 immediate-early gene (Lewis *et al.*, 1993; Purewal *et al.*, 1994). The sole IE gene encodes a polypeptide of 1487 amino acids (Gray *et al.*, 1987a, Grundy *et al.*, 1987, Harty *et al.*, 1989) that exhibits extensive homology to ICP4 of HSV-1 and ORF62 of varicella-zoster virus (VZV) (Grundy *et al.*, 1987). In transient transfection assays, the IE protein *trans*-activates EHV-1 and heterologous promoters, *trans*-represses its own expression, and acts synergistically with the EHV-1 ICP27 homolog to *trans*-activate early and late promoters (Matsumura *et al.*, 1993; Smith *et al.*, 1992, 1993; Zhao *et al.*, 1995). The IR2 gene is embedded within the IE gene and encodes an early protein that is a truncated (amino acids 323 to 1487) form of the IE protein (Harty and O'Callaghan, 1991). The IR2 protein lacks the *trans*-activation domain of the IE protein (residues 3 to 89) and is unable to *trans*-activate early and late promoters, but in transient transfection assays can *trans*-repress the IE promoter (Smith *et al.*, 1994; Kim *et al.*, submitted). The EHV-1 ICP0 homolog, ORF63, is regulated as an early gene, exhibits a low level of homology to HSV-1 ICP0, and retains the zinc RING finger found in the HSV-1 ICP0 protein (Telford *et al.*, 1992; Bowles and O'Callaghan, unpublished data; Everett *et al.*, 1993). The UL3 protein (ICP27 homolog) upregulates the expression of the IE promoter, but cannot overcome the downregulation that the IE protein exerts on its own expression (Zhao *et al.*, 1995). Although the UL3 protein does not efficiently *trans*-activate early and late promoters, the UL3 protein can act synergistically with the IE protein to increase expression from both early and late promoters (Zhao *et al.*, 1995).

The IR4 protein of 293 amino acids (Holden *et al.*, 1992b) exhibits homology to ICP22 of HSV-1 (McGeoch *et al.*, 1985), IEP-55 of BHV-1 (Schwyzer *et al.*, 1994), ORF63 of varicella-zoster (VZV) (Scott and Davis, 1986), IR2 of simian varicella-zoster virus (SVV) (Gray *et al.*, unpublished data), Rsp40 of pseudorabies virus (PRV)

[1] To whom correspondence should be addressed. Fax number (318) 675-5764. Email DCALLA@POP3.LSUMC.EDU.

0042-6822/95 $12.00

(Zhang and Leader, 1990), ORF4 of EHV-4 (Cullinane *et al.*, 1988), U_s ORF1 of Marek's disease virus (Sakaguchi *et al.*, 1993), and U_s ORF1 of herpesvirus of turkeys (Zelnik *et al.*, 1993). In previous work (Holden *et al.*, 1992b, 1994), the IR4 gene was shown to be differentially expressed as an early 1.4-kb transcript and a late 1.7-kb transcript, and its protein product was identified as a series of proteins that migrates from 42 to 47 kDa. During an EHV-1 lytic infection the IR4 protein is localized to the nucleus in a diffuse staining pattern and becomes packaged within EHV-1 virions (Holden *et al.*, 1994).

The present report addresses the functional importance of the IR4 protein in EHV-1 gene regulation and demonstrates that the IR4 protein: (i) minimally *trans*-activates EHV-1 promoters, (ii) acts *in trans* with the UL3 (ICP27) gene product to upregulate the IE promoter, (iii) cannot prevent the repression of the IE promoter by the IE protein, (iv) enhances *trans*-activation of early promoters by the IE protein, (v) enhances the *trans*-activation of early and late promoters by the IE and UL3 proteins, and (vi) interacts synergistically with the IE protein to *trans*-activate the heterologous HSV-1 ICP4 promoter.

MATERIALS AND METHODS

Cell culture. L-M cells were propagated in suspension cultures with YELP suspension medium (yeast extract, lactalbumin hydrolysate, peptide, and EMEM) containing 0.12% methyl cellulose, 100 mg/ml streptomycin, 100 mg/ml penicillin, and 5% fetal bovine serum.

Plasmids. The pSVIE, pSVUL3, and pcDR4 effector plasmids have been previously described (Smith *et al.*, 1992; Zhao *et al.*, 1995; Holden *et al.*, 1994). The pC12 expression vector which expresses the EHV-1 ORF12 protein was generated by cloning the ORF12 sequence into the pcDNA I Amp vector (Invitrogen; San Diego, CA), as described elsewhere in detail (J. B. Lewis *et al.*, manuscript in preparation). The EHV-1 IE, UL3, TK, and IR5 promoter–chlormaphenicol acetyltransferase gene chimeras have been described elsewhere (Smith *et al.*, 1992; Zhao *et al.*, 1995). The pPOH2 plasmid which contains the HSV-1 IPC4 promoter upstream of the CAT gene was a generous gift of Dr. Gary Hayward. To generate a gK–CAT chimeric construct, the pP2 (Zhao *et al.*, 1992) plasmid was digested with *Rsa*I, and the resultant 538-bp fragment was cloned into *Hin*cII-digested pUC19 to generate pUCL4. The pUCL4 plasmid was digested with *Hin*dIII and *Xba*I, and the resultant fragment was cloned into *Hin*dIII and *Xba*I digested pCAT–Basic plasmid (Promega; Madison, WI). The gK–CAT chimeric construct contains the −524 to +14 sequences of the EHV-1 UL4 (glycoprotein K [gK]) gene. The pIR4(E)–CAT(−365 to +17) construct was generated by digesting pCS-1

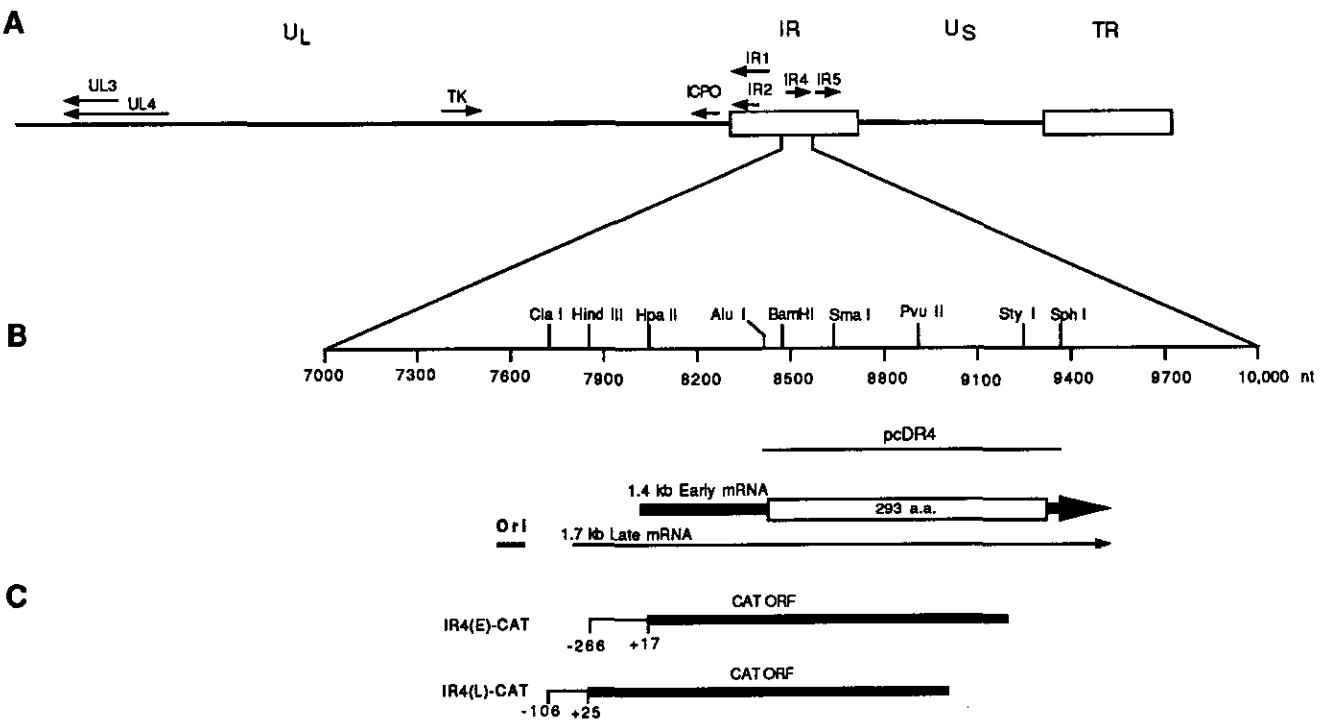

FIG. 1. IR4 expression construct and IR4 promoter–CAT constructs. (A) Diagram of the EHV-1 (KyA strain) genome. The locations of the UL3 (ICP27), UL4 (gK), thymidine kinase (TK), ICP0, IE (ICP4), IR2, IR4 (ICP22), and IR5 (US10) genes are shown by arrows. (B) The IR4 region (nt 7000 to 10,000) is expanded, showing pertinent restriction sites and the IR4 expression construct pcDR4 which contains the IR4 open reading frame of 293 aa (nt 8446 to 9327). The IR4 gene region is diagrammed, showing the location of the origin of replication (Ori_S)) and the two IR4 transcripts that have previously been mapped. (C) Diagram of the two IR4 promoter–CAT constructs. The IR4 promoter–CAT constructs were generated by cloning the IR4 promoters upstream of the chloramphenicol acetyltransferase (CAT) open reading frame within the pCAT–Basic vector (Promega).

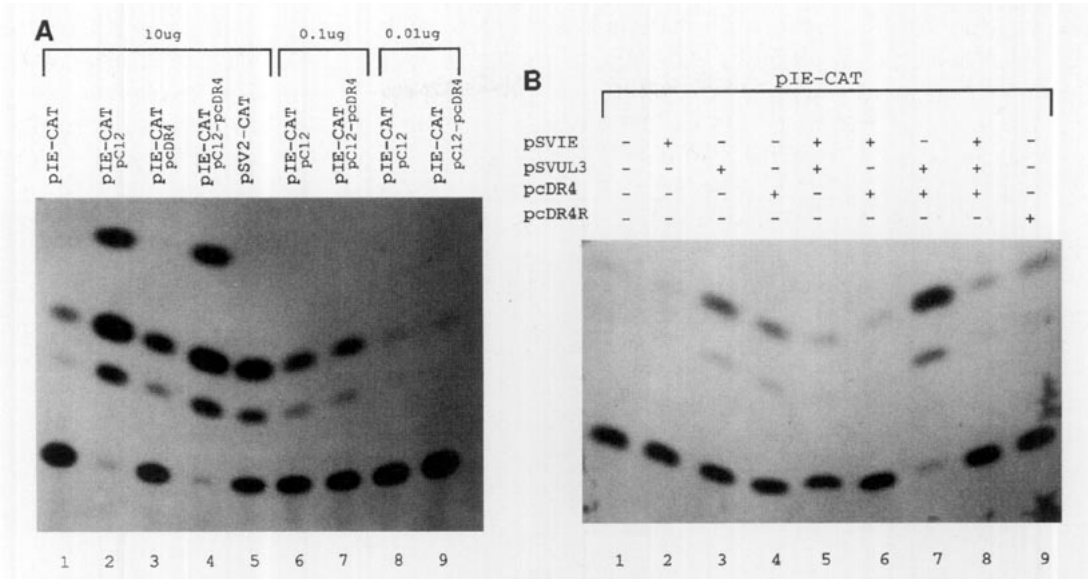

FIG. 2. Response of the EHV-1 IE (ICP4) promoter to the gene products of IR4 (ICP22), UL3 (ICP27), ORF12 (α-TIF), and IE (ICP4). (A) The EHV-1 pIE–CAT plasmid (1.4 pmol) was transfected into L-M cells in the presence or absence of 0.3 pmol of pC12 (α-TIF) and 0.3 pmol of pcDR4 (ICP22). The CAT assay in lanes 1 through 5 were performed using 10 μg of total protein, lanes 6 and 7 were performed with 0.1 μg of total protein, and lanes 8 and 9 were performed with 0.01 μg of total protein. The CAT assays performed in lanes 6 and 8, and 7 and 9 correspond to the same sample as that of lane 2 and 4, respectively. (B) L-M cells were transfected with 1.4 pmol of the pIE–CAT reporter plasmid and 0.3 pmol of various combinations of pSVIE, pSVUL3, and pcDR4 effector plasmids. Each transfection was performed in triplicate (Table 1).

(Holden *et al.*, 1992b) with *Hpa*II, and the resultant 374-bp fragment was cloned into *Acc*I-digested pCAT–Basic plasmid. In addition to containing the IR4 early promoter, this construct also contains portions of the IR4 late promoter. To remove the TATA box of the IR4 late promoter, pIR4(E)–CAT (−365 to +17) was digested with *Hin*dIII, and the vector was religated to generate the pIR4(E)–CAT (−266 to +17) construct. The pS1BH1 plasmid (Harty *et al.*, 1989) was digested with *Hin*dIII, and the resultant 1887-bp fragment was cloned into *Hin*dIII-digested pCAT–Basic plasmid to generate SR21. The SR21 plasmid was digested with *Cla*I and *Bam*HI, and the resultant IR4(L)–promoter–CAT chimeric construct was cloned into *Acc*I and *Bam*HI-digested pUC19 to generate pIR4(L)–CAT (−106 to +25) construct. The pIR4(L)–CAT chimeric construct does not contain the EHV-1 Ori_s sequences (Fig. 1).

Transfection procedure. L-M cells were plated at a density of 4×10^6 cells per tissue culture dish (60 mm) in EMEM (5% FBS), and allowed to attach overnight at 37° in a 5% CO_2 incubator. The cell monolayers were washed three times with serum-free EMEM, a final volume of 3 ml of serum-free EMEM was added to each plate, and liposome-mediated DNA transfection was performed using the Lipofectin reagent (Bethesda Research Laboratories). The DNA was combined with the Lipofectin reagent (5:1, w/w), incubated for 15 min, and added dropwise to the appropriate tissue culture dish. After 5 hr incubation, the medium was removed, EMEM medium containing 5% fetal bovine serum was added, and the cells were incubated an additional 60 to 62 hr. The pIE–CAT, pPOH2, pUL3–CAT, pTK–CAT, and pIR4(E)–CAT plasmids were transfected at 1.4 pmol amounts, and the EHV-1 late promoters pIR5–CAT, pUL4–CAT, and pIR4(L)–CAT plasmids were transfected at 2.0 pmol amounts. All effector plasmids (pSVIE, pSVUL3, pC12, and pcDR4) were transfected at 0.3 pmol amounts. The total amount of DNA applied per transfection was adjusted to 10 μg by the addition of pUC19.

CAT assays. CAT assays were performed essentially as described elsewhere (Rosenthal, 1987; Smith *et al.*, 1992). The L-M cells were washed three times with phosphate-buffered saline and scraped into TEN solution (40 m*M* Tris–HCl [pH 7.5], 10 m*M* EDTA, 150 m*M* NaCl), collected by centrifugation, and resuspended in 200 ml of 250 m*M* Tris–HCl (pH 8.0). The cells were lysed by freeze–thawing five times, and the cell lysates were heated at 60° for 10 min and clarified by centrifugation. The total protein concentration of each lysate was determined by using the BCA protein assay reagent (Pierce, Rockford Ill.). For each set of transfections, equal amounts of protein were assayed for CAT activity. The samples were adjusted to 158 μl using 250 m*M* Tris–HCl (pH 8.0), and 2 μl of [^{14}C]chloramphenicol (0.1 μCi, 50 to 60 mCi/mmol; New England Nuclear Corp., Boston, MA) and 20 μl of 3.5 mg/ml acetyl coenzyme A were added. The mixture was incubated at 37° for 60 min, chloramphenicol and its derivatives were extracted with ethyl acetate, and the ethyl acetate was evaporated. The dried samples were resuspended in 30 μl ethyl acetate, 15 μl was spotted onto thin-layer chromatography (TLC) sheets (silica gel IB; J. T. Baker), and chromatography

TABLE 1

Effects of EHV-1 ICP22 on the EHV-1 IE Promoter

Plasmids transfected	CAT activity[a] 1	2	3	Average % acetylation (mean ± SD)	Fold change[b] (mean)
10 μg[c]					
pIE(ICP4)-CAT	10.9	1.33	4.7	5.6 ± 4.9	
+pC12	99.3	99.1	99.1	99.2 ± 0.1	17.6
+pcDR4	38.4	40.4	15.4	31.4 ± 13.9	5.6
+pC12 + pcDR4	99.5	99.2	99.1	99.3 ± 0.2	17.6
pSV2-CAT	27.9	81.9	39.5	49.8 ± 28.4	
0.1 μg[c]					
pIE-CAT					
+pC12	8.2	7.93	10.7	8.9 ± 1.5	
+pC12 + pcDR4	5.4	8	ND	6.7 ± 1.8	
0.01 μg[c]					
pIE-CAT					
+pC12	0.97	1.06	1.16	1.1 ± 0.1	
+pC12 + pcDR4	2.06	0.68	1.09	1.3 ± 0.7	
pIE(ICP4)-CAT[d]	5.31	3.84	7.75	5.6 ± 2	
+pSVIE	2.31	1.84	2.03	2.1 ± 0.2	0.4
+pSVUL3	30.9	16.8	58.7	35.5 ± 21	6.3
+pcDR4	16.8	14.8	18.3	16.6 ± 1.7	3.0
+pSVIE + pSVUL3	7.4	10.6	6.27	8.1 ± 2.3	1.4
+pSVIE + pcDR4	3.48	14.7	13.3	10.5 ± 6.1	1.8
+pSVUL3 + pcDR4	97.4	83.3	85.9	88.9 ± 7.5	15.8
+psVIE + pSVUL3 + pcDR4	7.35	11	10.7	9.7 ± 2	1.7
+pcDR4R	9.91	4.95	10.9	8.6 ± 3.2	1.5

[a] Determined as percentage of acetylation. Results of independent transfection experiments are shown. ND, not done.

[b] Determined by comparison of the percentage acetylation values associated with the target plasmid in the presence of the indicated effector plasmid(s) versus the average percentage acetylation in the absence of effector plasmid(s). pSVIE(ICP4); pSVUL3(ICP27); pcDR4(ICP22); pC12(α-TIF).

[c] Each reaction was performed with the indicated amount of protein.

[d] Each reaction was performed using 80 μg of protein.

was performed for 2 hr in TLC tanks containing chloroform–methanol (19:1, v/v). The sheets were air dried and exposed (−70°) to Kodak X-Omat XAR-5 film. Chloramphenicol and its acetylated derivatives were excised from the silica gel, and the radioactivity associated with each spot was quantitated by liquid scintillation counting.

RESULTS

Effect of the IR4 protein on the EHV-1 immediate-early promoter. Since the IR4 protein is packaged in EHV-1 virions (Holden *et al.*, 1994), it was important to determine whether the EHV-1 IR4 protein can function as a *trans*-acting factor that affects expression of the EHV-1 immediate-early promoter (Fig. 1). The ORF12 protein, an HSV-1 α-TIF homolog (Telford *et al.*, 1992), has been shown to be a strong *trans*-activator of the EHV-1 IE promoter (Lewis *et al.*, 1993; Purewal *et al.*, 1994), and studies were undertaken to determine the whether ORF12 and IR4 proteins could act synergistically to *trans*-activate the IE promoter. The IR4 expression vector, pcDR4 (Fig. 1), was cotransfected into L-M cells with a pIE-CAT chimeric construct alone or in combination with the ORF12 expression plasmid (pC12) (Fig. 2A and Table 1). The results of this experiment demonstrated that the IR4 protein increased CAT activity by 5.6-fold compared to a 17.6-fold increase by the ORF12 protein. The coexpression of both ORF12 and IR4 proteins resulted in no increase in CAT activity compared to that of ORF12 alone; however, since all the substrate was utilized in this experiment, the reactions were repeated using lessor amounts of protein (0.1 and 0.01 mg). These results (Fig. 2A, lanes 6, 7, 8, and 9; and Table 1) revealed that the IR4 protein does not act synergistically with the ORF12 protein. The pSV2CAT plasmid contains the CAT gene driven by the SV40 early promoter and enhancer (Gorman *et al.*, 1982) and was used as a positive control.

In a separate experiment the IR4 protein was coexpressed with the EHV-1 IE and UL3 proteins to characterize further the ability of the IR4 protein to *trans*-activate the EHV-1 IE promoter. Since earlier studies (Smith *et al.*, 1992, 1994) demonstrated that the IE protein *trans*-represses its own promoter, the pcDR4 and pSVIE plasmids were cotransfected to determine whether the IR4 protein could affect the downregulation of the IE promoter. The results revealed that the IR4 protein is unable

TABLE 2

Effects of EHV-1 ICP22 on the EHV-1 Early Promoters

Plasmids transfected	CAT activity[a] 1	2	3	Average % acetylation (mean ± SD)	Fold change[b] (mean)
UL3(ICP27)-CAT	0.09	0.1	0.11	0.1 ± 0.01	
+pSVIE	5.9	1.1	1.2	2.7 ± 2.7	27.3
+pSVUL3	0.59	0.44	0.38	0.5 ± 0.1	4.7
+pcDR4	0.47	0.57	0.4	0.5 ± 0.9	4.8
+pSVIE-pSVUL3	9.67	22.2	61.4	31.1 ± 27	311
+pSVIE-pcDR4	12.7	13.9	22.9	16.5 ± 5.6	165
+pSVUL3-pcDR4	26.4	20.6	28.7	25.2 ± 4.2	252
+pSVIE-pSVUL3-pcDR4	34.7	60.8	34.3	43.3 ± 15.2	433
pSV2-CAT	96.5	98	98.3	97.6 ± 1.0	
TK2-CAT	0.9	1.36	2.05	1.4 ± 0.6	
+pSVIE	9.48	9.36	14.6	11.1 ± 3	7.7
+pSVUL3	4.41	8.48	13.9	8.9 ± 4.8	6.2
+pcDR4	0.63	0.88	1.88	1.1 ± 0.7	0.8
+pSVIE-pSVUL3	75.7	68.4	51.4	65.2 ± 12.5	45.3
+pSVIE-pcDR4	17	27.4	28.1	24.2 ± 6.2	16.8
+pSVUL3-pcDR4	10.1	7.23	14.5	10.6 ± 3.7	7.4
+pSVIE-pSVUL3-pcDR4	97.7	97.3	96.9	97.3 ± 0.4	67.6
pSV2-CAT	91.6	87.8	82	87.1 ± 4.8	
IR4E(ICP22)-CAT	0.74	0.79	1.1	0.9 ± 0.2	
+pSVIE	8.25	8.75	7.26	8.1 ± 0.8	9.2
+pSVUL3	2	4.22	4.27	3.5 ± 1.3	4.0
+pcDR4	4.36	5.57	6.56	5.5 ± 1.1	6.3
+pSVIE-pSVUL3	19.5	19.9	25.5	21.6 ± 3.5	24.5
+pSVIE-pcDR4	48.6	63.6	28.4	46.9 ± 17.7	53.3
+pSVUL3-pcDR4	5.09	7.04	4.56	5.7 ± 1.3	6.3
+pSVIE-pSVUL3-pcDR4	72.1	49.5	81.5	67.7 ± 16.5	76.9
pSV2-CAT	85.2	89	69	81.1 ± 10.6	

[a] Determined as percentage of acetylation. Results of independent transfection experiments are shown. Each reaction was performed using 80 μg of protein.

[b] Determined by comparison of the percentage acetylation values associated with the target plasmid in the presence of the indicated effector plasmid(s) versus the average percentage acetylation in the absence of effector plasmid(s). pSVIE(ICP4); pSVUL3(ICP27); pcDR4(ICP22).

to relieve the downregulation of the IE protein (Table 1). This finding is not unexpected since our recent studies reveal that autoregulation of the IE gene is mediated by IE amino acid sequences mapping at amino acid 422 to 597 which bind to the IE promoter and prevent transcription (Kim *et al.*, unpublished data). Sequences related to this autoregulatory domain of the IE protein are not present in the IR4(ICP22) protein. Individually, both the UL3 (ICP27) protein and the IR4 (ICP22) protein increased expression of the IE promoter to levels of 6.3 and 3 fold, respectively (Fig. 2B, lanes 3 and 4; Table 1); however, cotransfection of the UL3 and IR4 expression constructs enhanced expression of the IE promoter to a level of 15.8-fold (Fig. 2B, lane 7; Table 1). Thus, these two virion-associated proteins (Holden *et al.*, 1994; Y. Zhao *et al.*, unpublished data) act synergistically to enhance expression of the IE promoter, but do not prevent IE downregulation as cotransfection with the pSVIE construct reduced the 15.8-fold increase to a level of 2-fold (Fig. 2B, lanes 7 and 8; Table 1). The pcDR4R construct which contains the IR4 ORF in the reverse orientation downstream of the human cytomegalovirus major immediate-early promoter–enhancer (Holden *et al.*, 1994) was used as a negative control. In this experiment the pcDR4R construct gave a 1.5-fold activation, but in duplicate experiments (data not shown), the pcDR4R construct yielded levels equal to that of IE-CAT alone.

Effect of the IR4 protein on EHV-1 early promoters. The pcDR4 plasmid was cotransfected with three representative EHV-1 early promoter–CAT chimeric constructs (pUL3–CAT[ICP27], pTK–CAT, and pIR4(E)–CAT[ICP22]) into L-M cells alone or in combination with the pSVIE and pSVUL3 effector plasmids (Fig. 3, and Table 2) to determine the ability of the IR4 protein to act *in trans* on EHV-1 early promoters. Smith *et al.* (1992) demonstrated that the EHV-1 IE protein could efficiently *trans*-activate EHV-1 early promoters. In contrast, the IR4 protein alone does not *trans*-activate early promoters as efficiently as the IE protein (Fig. 3, lane 4; Table 2). However, when pcDR4 (an IR4 expression plasmid) is cotransfected with pSVIE and pUL3–CAT, a 165-fold increase in CAT activity is observed (Fig. 3, lanes 1, 2, and 6; Table 2) compared

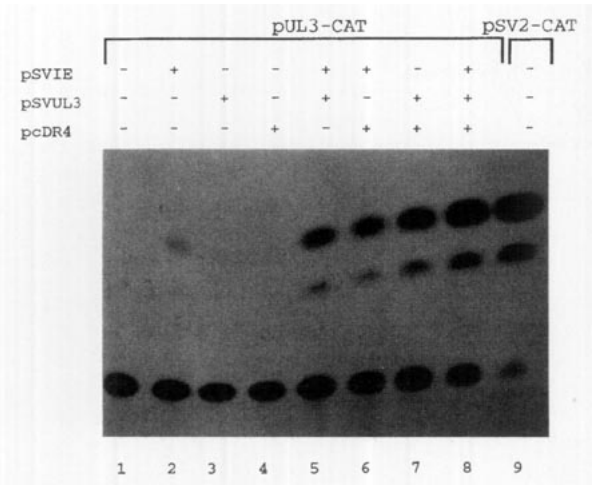

FIG. 3. Response of EHV-1 pUL3 (ICP27)-CAT construct to the gene products of IR4 (ICP22), IE (ICP4), and UL3 (ICP27). L-M cells were transfected with 1.4 pmol of the pUL3–CAT reporter plasmid and 0.3 pmol of various effector plasmids. Transfected cells were harvested approximately 62 hr post-transfection, and CAT activity was assayed. Each transfection was performed in triplicate (Table 2).

to the 27.3-fold activation with the IE protein alone. The UL3 protein alone only minimally activates (4.7-fold) the UL3 promoter (Fig. 3, lane 3; Table 2). Interestingly, cotransfection of pcDR4 and pSVUL3 (an UL3 expression plasmid) resulted in a 252-fold increase in CAT activity expressed from the UL3 promoter (Fig. 3, lanes 7; Table 2), indicating that this early promoter does not require the IE protein for high levels of its expression. Previous work has shown that the UL3 protein acts synergistically with the IE protein to increase UL3–CAT activity (Zhao *et al.*, 1995). As shown here, expression of the IR4, IE, and UL3 proteins results in a 433-fold increase in CAT activity compared to that of 311-fold increase with the IE and UL3 proteins alone (Fig. 3, lanes 5 and 8; Table 2). Similarly, as had been shown in the case of the EHV-1 UL3 (ICP27) promoter, the IR4 protein acts *in trans* with the IE protein to increase CAT activity controlled by the early promoters of the TK gene (TK-CAT; 16.8-fold) and the IR4 gene (IR4E-CAT; 53.3-fold) (Table 2). Moreover, the IR4 protein increases the enhanced expression of the pTK–CAT (67.6-fold) and pIR4(E)–CAT (76.9-fold) chimeric genes mediated by the IE and UL3 gene products (Table 2). However, the IR4 and UL3 proteins in the absence of the IE gene product do not act synergistically on the TK–CAT and IR4(E)–CAT chimeric genes (Table 2). Overall, the above findings revealed that the IR4 protein alone is not able to efficiently activate EHV-1 early promoters, that the IR4 protein acts *in trans* with the IE protein to increase expression of EHV-1 early promoters, and that the IR4 protein can act *in trans* with both the IE and UL3 proteins to increase expression of EHV-1 early promoter activity.

Effects of the IR4 protein on EHV-1 late promoters. To assess the ability of the IR4 protein to *trans*-activate EHV-1 late promoters, the pcDR4 construct was cotransfected with IR5(US10)–CAT, IR4(L)–CAT, or gK–CAT, and in combination with the pSVIE and pSVUL3 effector plasmids. Expression of the IR4 protein alone did not efficiently *trans*-activate the IR5–CAT or gK–CAT construct (Fig. 4, lane 4; Table 3). Moreover, the IR4 protein did not act synergistically with either the IE protein or the UL3 protein to *trans*-activate the IR5-CAT or gK–CAT constructs (Fig. 4, lanes 6 and 7). However, when expressed with both the IE and UL3 proteins, the IR4 protein did significantly increase CAT activity from the IR5–CAT construct (Fig. 4, lane 8, Table 3). The gK–CAT construct was not significantly activated when all three proteins were expressed (Table 3). Although the IR4 protein when expressed alone did not efficiently *trans*-activate the IR5–CAT and gK–CAT chimeric constructs, a 10-fold activation was observed when pcDR4 was cotransfected with the IR4(L)–CAT chimeric construct. A significant increase in IR4(L) promoter activity was observed (Table 3) when the IR4 protein was expressed with either the IE protein (70.2-fold) or UL3 protein (49.6-fold), or with both proteins (142-fold). Interestingly, the IR4(L)–CAT construct (Fig. 1) which possesses characteristics of a γ-1 gene (Holden *et al.*, 1992b), acts as an early promoter in transient transfection assays.

Effect of the IR4 protein on the heterologous HSV-1 ICP4 promoter. The pcDR4 vector was cotransfected with the HSV-1 ICP4–CAT construct (pPOH2) to assess the ability of the IR4 protein to act *in trans* on a heterologous promoter. In contrast to its effect on the EHV-1 IE promoter, the IR4 protein does not upregulate the ICP4–CAT construct (Fig. 5, lane 3; Table 4). The IE protein as previously demonstrated (Smith *et al.*, 1992) can act *in trans* on the ICP4–CAT construct (Fig. 5, lane 2; Table 4). The cotransfection of both the pcDR4 and pSVIE effector

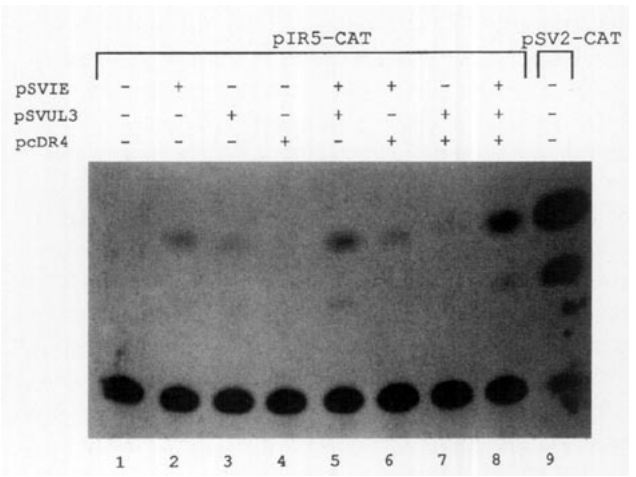

FIG. 4. Response of EHV-1 pIR5 (US10)–CAT reporter construct to the gene products of IR4 (ICP22), IE (ICP4), and UL3 (ICP27). L-M cells were transfected with 2.0 pmol of the pIR5–CAT reporter plasmid and 0.3 pmol of various effector plasmids. Transfected cells were harvested at 62 hr post-transfection, and CAT activity was assayed. Each transfection was performed in triplicate (Table 3).

TABLE 3

Effects of EHV-1 ICP22 on the EHV-1 Late Promoters

Plasmids transfected	CAT activity[a] 1	2	3	Average % acetylation (mean ± SD)	Fold change[b] (mean)
IR5(US10)-CAT	0.28	0.22	0.33	0.3 ± 0.1	
+pSVIE	2.49	0.35	0.31	1.1 ± 1.3	3.8
+pSVUL3	1.15	0.61	1.28	1.0 ± 0.4	3.6
+pcDR4	0.39	0.52	0.53	0.5 ± 0.1	1.7
+pSVIE-pSVUL3	4.2	2.74	1.41	2.8 ± 1.4	9.9
+pSVIE-pcDR4	1.05	1.01	0.98	1.0 ± 0.1	3.6
+pSVUL3-pcDR4	0.71	0.47	0.48	0.6 ± 0.1	2.0
+pSVIE-pSVUL3-pcDR4	6.53	3.18	5.06	4.9 ± 1.7	17.6
pSV2-CAT	95.7	93.8	85.2	91.6 ± 5.6	
IR4L(ICP22)-CAT	0.34	0.51	0.49	0.5 ± 0.1	
+pSVIE	15.7	14.3	8.5	12.8 ± 3.8	28.4
+pSVUL3	9.17	8.1	9.64	9.0 ± 0.8	19.9
+pcDR4	4.56	7.34	2.45	4.8 ± 2.5	10.6
+pSVIE-psVUL3	50.9	73.9	40.9	55.2 ± 17	123
+pSVIE-pcDR4	24.2	27.2	43.5	31.6 ± 10	70.2
+pSVUL3-pcDR4	12.9	34.4	19.6	22.3 ± 11	49.6
+pSVIE-pSVUL3-pcDR4	79.2	55.3	57.2	63.9 ± 13	142
pSV2-CAT	88.6	28.9	66.5	61.3 ± 30	
gK-CAT	0.21	0.25	0.35	0.3 ± 0.1	
+pSVIE	0.75	0.53	0.51	0.6 ± 0.1	2.2
+pSVUL3	0.79	0.94	0.45	0.7 ± 0.2	2.7
+pcDR4	0.72	0.59	0.39	0.6 ± 0.2	2.1
+pSVIE-pSVUL3	1.11	1.09	0.87	1.0 ± 0.1	3.8
+pSVIE-pcDR4	0.41	0.51	0.31	0.4 ± 0.1	1.5
+pSVUL3-pcDR4	1.13	1.02	0.68	0.9 ± 0.2	3.5
+pSVIE-pSVUL3-pcDR4	1.74	2.49	2.02	2.1 ± 0.4	7.7
pSV2-CAT	89.3	76.4	84.9	83.5 ± 6.6	

[a] Determined as percentage of acetylation. Results of independent transfection experiments are shown. Each reaction was performed using 80 μg of protein.

[b] Determined by comparison of the percentage acetylation values associated with the target plasmid in the presence of the indicated effector plasmid(s) versus the average percentage acetylation in the absence of effector plasmid(s). pSVIE(ICP4); pSVUL3(ICP27); pcDR4(ICP22).

plasmids with the pPOH2 plasmid resulted in a 6.7-fold increase in CAT activity (Fig. 5, lane 4; Table 4), indicating that the IR4 and IE proteins can act synergistically to upregulate the HSV-1 ICP4 promoter.

DISCUSSION

We have previously identified the IR4 gene as a homolog of HSV-1 ICP22 and documented its differential expression as early and late transcripts (Holden *et al.*, 1992b). The IR4 protein is localized to the nucleus in the EHV-1-infected cell and is packaged within EHV-1 virions (Holden *et al.*, 1994). As demonstrated in this report, the IR4 protein alone is not a strong *trans*-activator, but significantly enhances the upregulation of EHV-1 chimeric promoter-CAT reporter constructs when expressed in combination with the EHV-1 IE(ICP4) protein and the UL3(ICP27) protein.

Genes encoding ICP22 proteins have been identified in nine α-herpesviruses (Holden *et al.*, 1992b; Cullinane *et al.*, 1988; Davison and Scott, 1986; W. L. Gray *et al.*, unpublished data; McGeoch *et al.*, 1985; Schwyzer *et al.*, 1994; Sakaguchi *et al.*, 1993; Zelnik *et al.*, 1993; Zhang and Laeder, 1990). Based on an amino acid alignment of the ICP22 homologs, Schwyzer *et al.* (1994) proposed that the ICP22 homologs contain four distinct regions. Region 1 corresponds to the N-terminal 1–64 amino acids of the IR4 protein, varies in length among the ICP22 homologs, and shows little conservation of amino acid residues among the ICP22 proteins. Region 2 corresponds to residues 65 to 196 of the IR4 protein, and possesses the highest level of amino acid conservation among the ICP22 homologs. Preliminary findings suggest that region 1 and 2 of the IR4 protein are required for the synergistic interaction of the IR4 protein and the IE protein because deletions in region 1 and 2 render the IR4 protein nonfunctional in transient transfection assays (Holden *et al.*, unpublished data). Region 3 (residues 197 to 268 of the IR4 protein) lacks any distinct pattern of amino acid conservation, but is rich in acidic and hydroxyl residues. Interestingly, the ICP22 homologs of herpesvirus of turkeys and Marek's disease virus lacks this

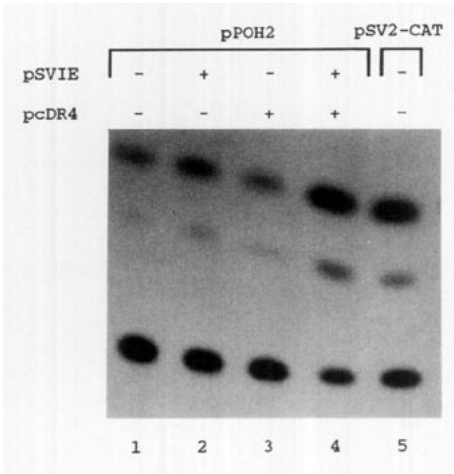

FIG. 5. Response of HSV-1 ICP4 promoter to the gene products of IR4 (ICP22) and IE (ICP4). L-M cells were transfected with 1.4 pmol of the pPOH2 (ICP4–CAT) reporter plasmid and 0.3 pmol of pSVIE and 0.3 pmol of pcDR4. Transfected cells were harvested at 62 hr post-transfection and CAT activity was assayed. Each transfection was performed in triplicate (Table 4).

acidic rich region (Sakaguchi *et al.*, 1993; Zelnik *et al.*, 1993). Since the IR4 protein is a *trans*-acting protein, region 3 is of interest because acidic domains of other regulatory proteins have been shown to be important for *trans*-activation (Cress and Triezenberg, 1991). Finally, region 4 (residues 269 to 293 of the IR4 protein) is a small region rich in basic residues (48%). This region of the IR4 protein, resembles a nuclear localization domain, and has been shown to be important for the nuclear localization of the EHV-1 IR4 protein (Holden *et al.*, 1994).

The ICP22 gene of HSV-1 is nonessential for virus replication in tissue culture (Poffenberger *et al.*, 1993; Post and Roizman 1981; Sears *et al.*, 1985). Studies by Sears *et al.* (1985) revealed that an $\alpha 22^-$ virus could replicate in human (HEp-2) and primate (Vero) cells at levels equivalent to those for the wild-type virus; however, virus plating efficiency and virus yield of the $\alpha 22^-$ virus were reduced in cell lines (BHK and RAT-1) of rodent origin. In the BHK and RAT-1 cells, the $\alpha 22^-$ virus exhibited prolonged expression of β proteins and a decrease in expression of γ proteins. Recent studies by Purves *et al.* (1993) revealed a decrease in mRNAs for the immediate-early ICPO gene and the late U_s11 gene in BHK cells infected with the $\alpha 22^-$ mutant, suggesting that ICP22 might function at the transcriptional level, or post-transcriptionally by stabilizing viral mRNAs. Biologically, the $\alpha 22^-$ virus is avirulent in mice (Sears *et al.*, 1985), but can establish a latent infection and can be reactivated from latently infected ganglia (Meignier *et al.*, 1987; Sears *et al.*, 1985).

The ICP22 proteins of EHV-1, BHV-1, and HSV-1 have been partially characterized (Ackermann *et al.*, 1985; Blaho *et al.*, 1993; Purves *et al.*, 1992, 1993; Holden *et al.*, 1994; Hayes and Rock, 1990; Wilcox *et al.*, 1980). All three proteins have been shown by cellular fractionation studies to localize in the nucleus (Hayes and Rock, 1990; Holden *et al.*, 1994; Wilcox *et al.*, 1980). Moreover, the EHV-1, IR4 protein as revealed by indirect immunofluorescence and laser scanning confocal microscopy studies displays a diffuse nuclear staining pattern (Holden *et al.*, 1994). The EHV-1, BHV-1, and HSV-1 ICP22 proteins have predicted molecular weights of 32.8, 30.9, and 46.5 kDa, respectively; however, these proteins migrate as 42–47 (EHV-1), 52–57 (BHV-1), and 68–72 (HSV-1) kDa. This discrepancy in the predicted size and apparent size of the ICP22 homologs might result from the unusual amino acid content and/or post-translation modifications. Both the BHV-1 and HSV-1 ICP22 proteins are extensively phosphorylated (Hayes and Rock, 1990; Wilcox *et al.*, 1980; Purves and Roizman 1992), and the HSV-1 ICP22 protein is also guanylyated and adenylyated (Blaho *et al.*, 1993).

The role that the ICP22 protein or its homologs plays in gene regulation has not yet been ascertained. Although Purves *et al.* (1993) demonstrated that the ICP22 protein directly or indirectly affects the level of mRNAs for ICPO and U_s11 genes, the ICP22 protein in transient transfection assays does not demonstrate detectable *trans*-act-

TABLE 4

Effects of EHV-1 ICP22 on the HSV-1 ICP4 Promoter

Plasmids transfected	CAT activity[a] 1	2	3	Average % acetylation (mean ± SD)	Fold change[b] (mean)
pPOH2 (ICP4)	14.6	10.4	3.99	9.6 ± 5.3	
+pSVIE	24.2	14.9	5.95	15 ± 9.1	1.6
+pcDR4	15	5.5	3.05	7.8 ± 6.3	0.8
+pSVIE + pcDR4	73.4	66.5	53.3	64.4 ± 10.2	6.7
pSV2-CAT	57	58.8	74.9	63.6 ± 9.8	

[a] Determined as percentage of acetylation. Results of independent transfection experiments are shown. Each reaction was performed with 80 μg of protein.

[b] Determined by comparison of the percentage acetylation values associated with the target plasmid in the presence of the indicated effector plasmid(s) versus the average percentage acetylation in the absence of effector plasmid(s). pSVIE(ICP4); pcDR4(ICP22).

ing ability for HSV-1 promoters either alone or in combination with other HSV-1 immediate-early proteins (Everett, 1984; Gelman and Silverstein, 1985; Mavromara-Nazos *et al.*, 1986; O'Hare and Hayward, 1985). In contrast to the HSV-1 ICP22 protein, the VZV ICP22 protein (ORF63) possesses regulatory capabilities in that the ORF63 protein *trans*-represses the VZV ORF62(ICP4) promoter (Jackers *et al.*, 1992). However, the ORF63 protein does not affect expression of the promoters of two late genes, glycoprotein I and II. Studies have not been published as to the ability of the ORF63 protein to act in combination with other VZV regulatory proteins to *trans*-activate VZV promoters. The results presented here show that the EHV-1 IR4 protein is involved in EHV-1 gene regulation, and future investigations should reveal whether this protein functions at the transcriptional and/or post-transcriptional level.

ACKNOWLEDGMENTS

We thank Suzanne Zavecz and Scarlett Flowers for excellent technical support and Dr. John Staczek for critical reading of the manuscript. This investigation was supported by Public Health Research Grant AI 22001 to D.J.O. from the National Institutes of Health, and Grants 89-37266-4735 and 92-37204-8040 from the U.S. Department of Agriculture Animal Molecular Biology Program to D.J.O.

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